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Project Entitled: ***Biology of Somatostatin and Somatostatin Receptors in Breast Cancer***

Final Addendum Report For Award #DAMD17-96-1-6189

Introduction

The twelve tasks comprising this four year project were completed and a full report submitted in September 2000. However, in the course of these studies three new leads were pursued as three separate tasks (Tasks 13, 14, 15) which were not part of the original contract but because of their importance were nonetheless developed substantially and also reported upon in September 2000. We then requested and received approval for a no-cost extension to allow us to round off these three new tasks up to the period ending September 2001. We made reasonable progress in each of these three areas as outlined in this report despite the time and budgetary constraints and personnel departures. As emphasized in our Final Report, however, each of these three new tasks represented substantial new projects which we intend to pursue fully with renewed new longterm funding.

TASK 13 – Mutational Analysis of the C-Tail of hSSTR3

Having found that SSTR3 is the only of the five SSTRs that induces apoptosis when expressed as a monotransfectant, we undertook a mutational analysis of the role of the cytoplasmic tail of this receptor and created a series of C-tail deletion mutants as well as chimeric receptors described in Fig. 11 of the Final Report. These receptors were stably expressed in HEK293 cells to achieve comparable levels of expression. The kD and Bmax of the mutant receptors were similar to that of wild type receptors and like wild type receptors, the mutant receptors were all functionally coupled to inhibition of adenylyl cyclase. The number of viable cells were analysed by MTT assay and cells undergoing apoptosis were monitored by TUNEL and HOECHST assays. The principal finding to emerge from these studies was that deletion of the C-tail of SSTR3 abrogates SSTR3 induced apoptosis whereas substitution of the C-tail of SSTR5 (a nonapoptotic receptor) with that of SSTR3 confers apoptosis in the chimeric receptor clearly suggesting that apoptotic signalling by SSTR3 is dependent on molecular signals in the receptor C-tail. Our next objective was to identify these regulatory sequences and towards this we have constructed four C-tail deletion mutants - Δ 342, Δ 372, Δ 388, and Δ 415 (Fig. 1) which have been stably expressed in HEK293 cells and are currently being characterized for apoptotic signalling. Once we have narrowed down a putative regulatory sequence, we will use it as bait in a yeast 2 hybrid screen of a human fetal brain cDNA library to look for interacting proteins. The yeast 2 hybrid assay has been established as part of other funded projects in our laboratory and is fully functional. However, the screening and characterization of positive clones is a longterm undertaking and will require additional new funding.

Additional new studies pertinent to the role of SSTR3 as an apoptotic receptor were carried out as part of this task. In our Final Report, we showed that when SSTR1-5 are studied individually as monotransfectants, only SSTR3 induces apoptosis. In MCF7 cells which coexpress SSTR1, 2, 3, 5, however, treatment with subtype-selective agonists induces apoptosis via all four SSTRs. In light of our finding that SSTR subtypes can interact through heterodimerization, the differential ability of SSTR1, 2, 5 to induce apoptosis when coexpressed with SSTR3 but not when expressed alone, suggested that SSTR3 is an obligatory subtype for

SST-induced apoptosis, but that other SSTR subtypes can also induce apoptosis when coexpressed with SSTR3, likely through formation of SSTR3 heterodimers. Direct proof of this concept was obtained by studying stable cotransfectants of SSTR3/SSTR1, SSTR3/SSTR2, SSTR3/SSTR4, and SSTR3/SSTR5. We found induction of apoptosis (TUNEL assay) when treated with selective SSTR1, 2, 4, and 5 nonpeptide agonists in CHO-K1 cells coexpressing SSTRs with SSTR3 (Fig. 2). Since SSTR1, 2, 4, and 5 expressed as monotransfecants do not induce apoptosis and since SSTRs such as SSTR5 and SSTR1 have been reported by us to form functional heterodimers, the cotransfection experiments clearly raised the possibility of functional heterodimers between SSTR3 and the other members of this receptor family. We then applied photobleaching fluorescence resonance energy transfer (pbFRET) analysis using fluorescein and rhodamine-labelled anti-receptor antibody probes to investigate direct protein-protein interaction between the SSTR3 heterodimers. Treatment of the SSTR1/SSTR3 and SSTR2/SSTR3 cotransfectants with agonists selective for either receptor monomer induced FRET efficiencies of ~ 20% indicating physical association of the receptor pairs as heterodimers (Fig. 3). Treatment with the common agonist SST produced a comparable effect to that of the individual agonists. Similar results of pbFRET analyses were obtained in the case of the SSTR4/SSTR3 and SSTR5/SSTR3 cotransfectants. Interestingly, SSTR4 which is not expressed in MCF7 cells and which, therefore, did not induce apoptosis when treated with its agonist in these cells, was clearly able to induce apoptosis through heterodimerization with SSTR3 as a cotransfectant. Overall, the model that emerges (Fig. 4) suggests that SSTR3 is an essential receptor for mediating SST-induced apoptosis, but if other SSTR subtypes also coexist in the same cell, they can also induce apoptosis by forming heterodimers with SSTR3. This opens the way to a whole range of selective and nonselective SSTR subtype compounds for SST-induced apoptotic therapy.

TASK 14 – Mutational Analysis of the C-Tail of hSSTR5

Unlike SSTR3, SSTR5 inhibits cell growth by inducing cell growth arrest. C-tail trunkation mutants of hSSTR5 displayed progressive loss of antiproliferative signaling suggesting a crucial role of the C-tail domain of this receptor in cytostatic signaling. Since phosphorylation on serine and threonine residues plays an important role in G-protein coupled receptor regulatory functions such as effector coupling, agonist-dependent desensitization and internalization, we extended our study of the requirement of the C-tail of hSSTR5 in cytostatic signaling to an investigation of the role of phosphorylation sites within the C-tail. Seven mutant hSSTR5 receptors were constructed by PCR mutagenesis in which putative phosphorylation sites on threonine (T) and serine (S) residues were replaced by alanine (A) residues as follows: S 314A, S 325A, T333A, T 347A, T351A, T360A, and S361A. These mutants were described schematically in Fig. 16 of our Final Report and the pharmacological and antiproliferative signaling properties of the four threonine to alanine mutants were described. The T333A, T347A, and T360A mutants failed to transduce the antiproliferative signaling of SST whereas the T351A mutant displayed a > 5-fold weaker response to the anti-proliferative action of SST compared to wild type hSSTR5. To complete this task, we had proposed the characterization of the three S ➜ A mutants. S341A, S325A, and S361A mutant hSSTR5 receptors were expressed in CHO-K1 cells. All three cells, however, grew at a markedly decreased rate making it difficult to establish stable cell lines for pharmacological and cell growth analyses. We are still attempting to circumvent this difficulty but at the present time the role of the S ➜ A mutations in the C-tail of hSSTR5 remains incomplete. The results obtained so far with the T ➜ A substitution mutants, however, are dramatic and show a critical role of phosphorylation in cytostatic signaling by the C-tail of hSSTR5.

TASK 15 – Studies of cAMP Effects on SSTR Mediated Apoptosis

These studies have been completed in MCF7 cells. Our findings revealed that induction of cAMP through VIP receptor mediated activation or direct stimulation with forskolin of adenylyl cyclase prevented SST-induced apoptosis by attenuating SST-induced acidification. Treatment of these cells with dbcAMP also protected them from SST-induced cytotoxicity (Fig. 5). Moreover, dbcAMP did not prevent the SHP-1 mediated activation of caspase 8 (Fig. 6). These data suggested that cAMP acts downstream of caspase 8 to prevent SST-induced acidification. We proposed to investigate the protective effect of cAMP in hSSTR3 expressing CHO-K1 cells and have completed these experiments. In detailed time-course experiments we observed that the increase in cAMP prior to but not after the onset of acidification prevented hSSTR3-signaled apoptosis (Fig. 7). Since cAMP is known to phosphorylate and modulate the activity of the Na^+/H^+ exchanger (NHE) we predicted that SST regulates the functional behaviour of the NHE in a direction opposite to that triggered by cAMP. Moreover, the effect of cAMP appears to be that of protecting NHE but not rescuing it from the effect of SST. The existence of six isoforms of NHE and their variable expression in different cell types rendered the extension of these studies outside the scope of this grant. We have begun these new studies with separate funding from a different agency (Canadian Institutes of Health Research).

Legends to Figures

Figure 1: DNA and amino acid sequence of the cytoplasmic tail (C-tail) of the human SSTR3 (hSSTR3) subtype. The positions of the four C-tail deletion mutants [Δ342 (B1), Δ372 (C1), Δ388 (D1), and Δ415 (A1)] are shown. Hydrophobic residues are depicted in red font or circles. PBZ like motifs are underlined.

Figure 2: CHO-K1 cells stably coexpressing SSTR1 and SSTR3, SSTR2 and SSTR3, SSTR4 and SSTR3, and SSTR5 and SSTR3 were cultured in normal culture medium and treated with selective nonpeptide agonists to SSTR1, 2, 3, and 4 or with the common agonist SST as shown for the different paired combinations. Apoptosis was detected by TUNEL assay. Note the large number of rounded TUNEL positive apoptotic cells resulting from treatment with both the selective agonists as well as with SST.

Figure 3: pbFRET analysis of agonist-induced heterodimerization of SSTR3. SSTR1/SSTR3 and SSTR2/SSTR3 cell cotransfectants were grown in culture, treated with the selective nonpeptide agonists or with SST as shown (1 μm for 30 min) to induce ligand dependent receptor heterodimerization. The cells were then fixed and the receptor pairs labelled with fluorescein or rhodamine-tagged anti-SSTR antibodies. The cell membrane regions were then analysed on a pixel-by-pixel basis for photobleaching time constants and the delay in photobleaching in the presence of ligand expressed as percent FRET efficiency. Complete details of the technique have been described in earlier publications from our laboratory (Rocheville M. et al, *J. Biol. Chem. 275:7862, 2000*; Rocheville M. et al, *Science 288:154, 2000*).

Figure 4: Schematic model showing how multiple SSTRs can induce apoptosis through heterodimerization with SSTR3, the critical final common pathway essential for SST-dependent apoptosis.

SSTR3

C-TAIL **DNA SEQUENCE**

bp #		aa #
	tac cgc ttc	
961	aag cag ggc ttc cgc agg gtc ctg ctg cgg ccc tcc cgc cgt gtg cgc agc cag gag ccc	320 340
1021	act gtg [redacted] ggg ccc ccg gag aag act gag gag gag gat gag gag gag gag gat ggg gag gag	360
1081	agc agg gag ggg ggc aag ggg aag gag atg aac ggc cgg C1 gtc agc cag atc acg cag cct	380
1141	ggc acc agc ggg cag gag cgg ccg ccc agc [redacted] aga gtg gcc agc aag gag cag cag ctc cta	400
1201	ccc caa gag gct tcc act ggg gag aag tcc agc acg atg cgc atc A1 agc tac ctg tag C2	420

C-TAIL **AMINOACID SEQUENCE**

Name of construct	Position in the tail	Position in the receptor sequence	Amino acid
	[redacted]	(317)	VRFK QGF RR VLL R PSR RVR SQE PTV 25
[redacted]	[redacted]	(342)	GPP EKT EEE DEE EED GEE SRE GGK GKE MN 54
C1	[redacted]	(372)	GRV SQI T QPG TSG QER PPS 73
[redacted]	[redacted]	(388)	RVAS KE QQL LPQ EAS TGE KSS TMR I 98
A1	[redacted]	(415)	SYL
C2 (WT)			(418aa)

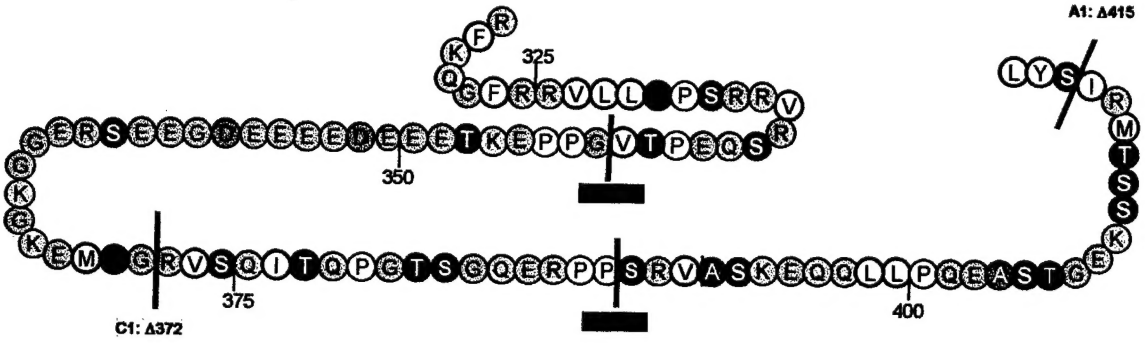

Figure 1

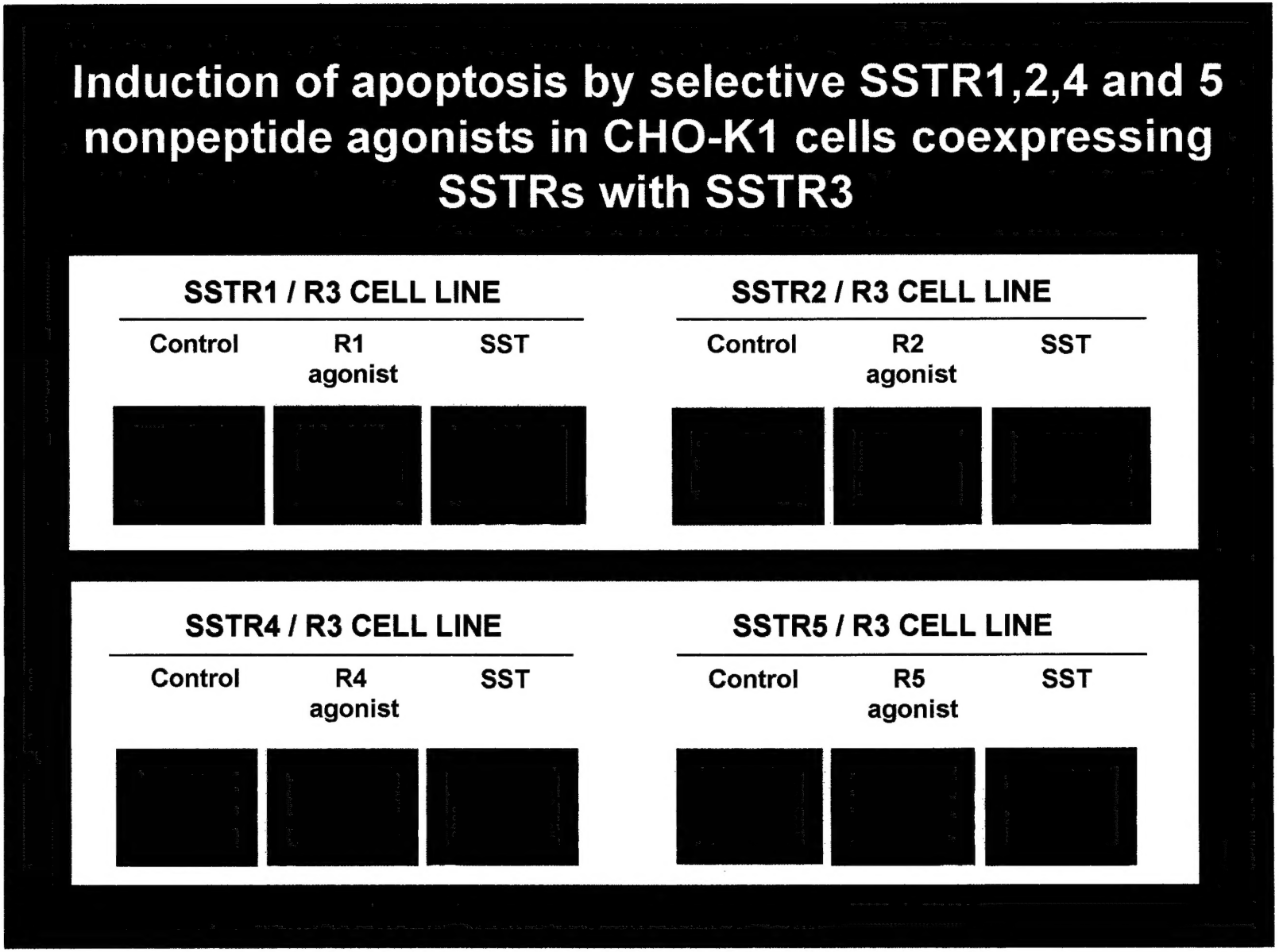
Induction of apoptosis by selective SSTR1,2,4 and 5 nonpeptide agonists in CHO-K1 cells coexpressing SSTRs with SSTR3
SSTR1 / R3 CELL LINE
Control
R1 agonist
SST
SSTR2 / R3 CELL LINE
Control
R2 agonist
SST
SSTR4 / R3 CELL LINE
Control
R4 agonist
SST
SSTR5 / R3 CELL LINE
Control
R5 agonist
SST

Figure 2

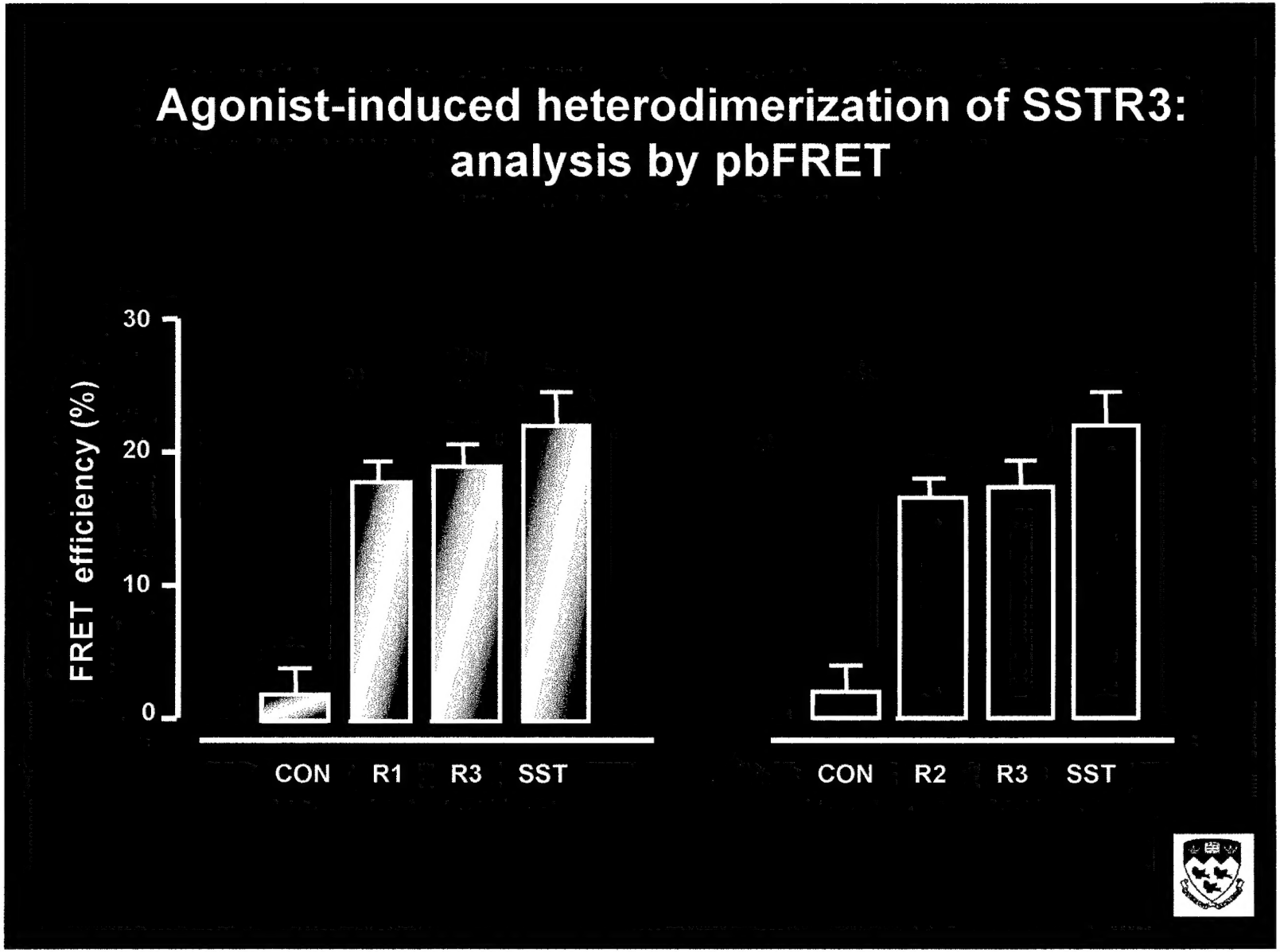
Agonist-induced heterodimerization of SSTR3: analysis by pbFRET
FRET efficiency (%)
30
20
10
0
CON
R1
R3
SST
CON
R2
R3
SST

Figure 3

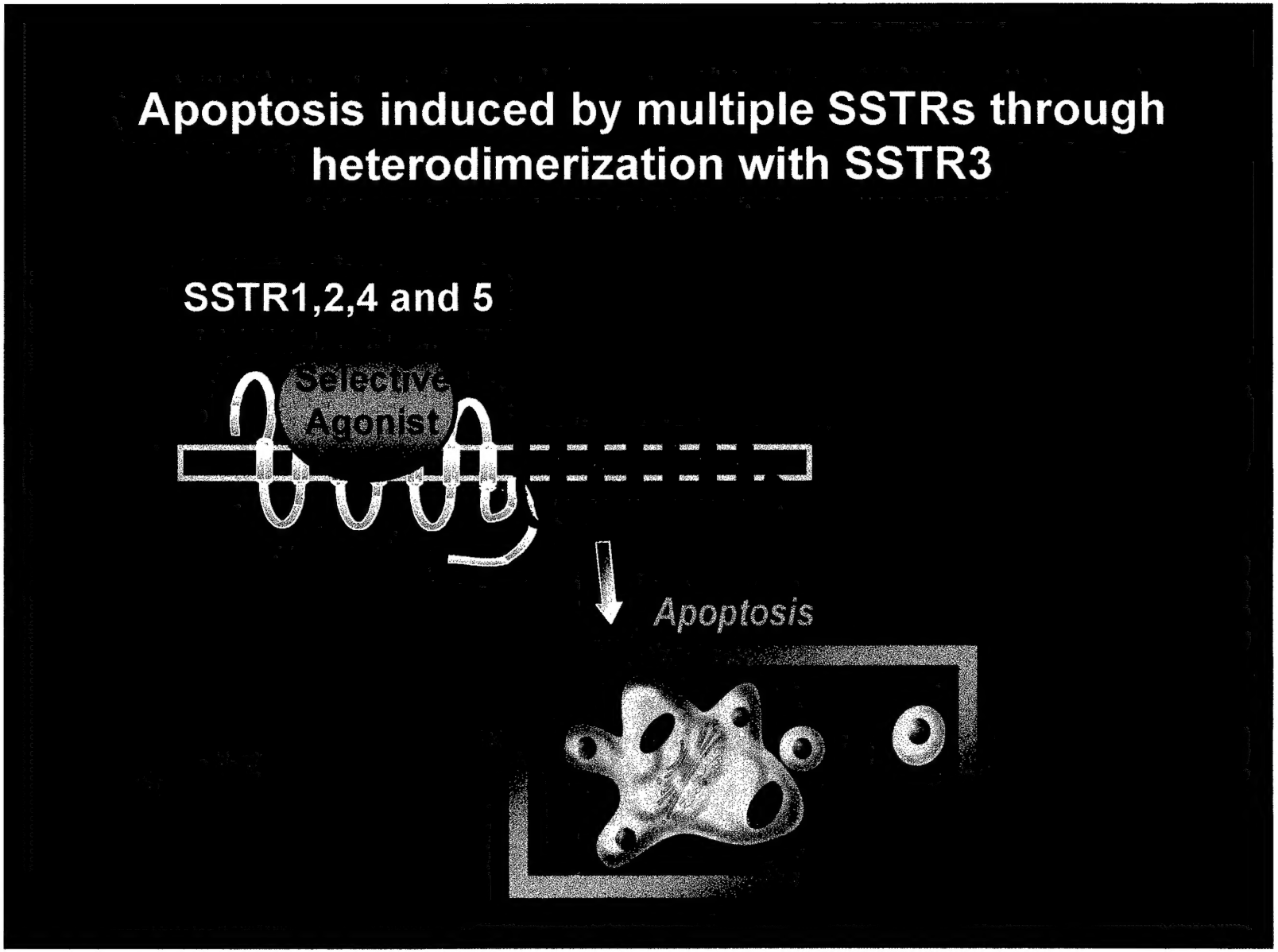
Apoptosis induced by multiple SSTRs through heterodimerization with SSTR3
SSTR1,2,4 and 5
Selective Agonist
Apoptosis

Figure 4

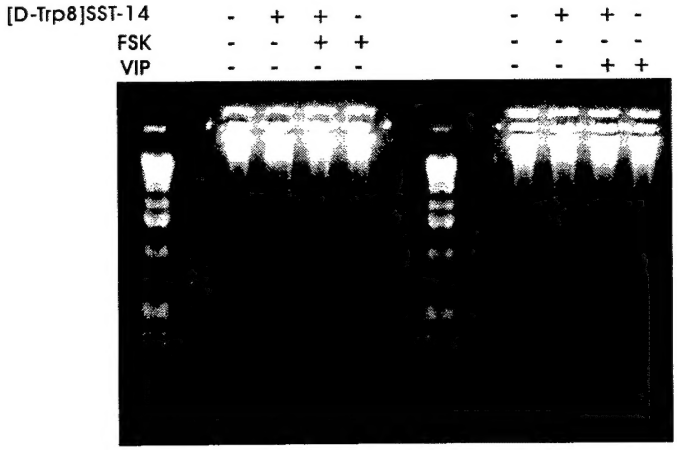

Figure 5

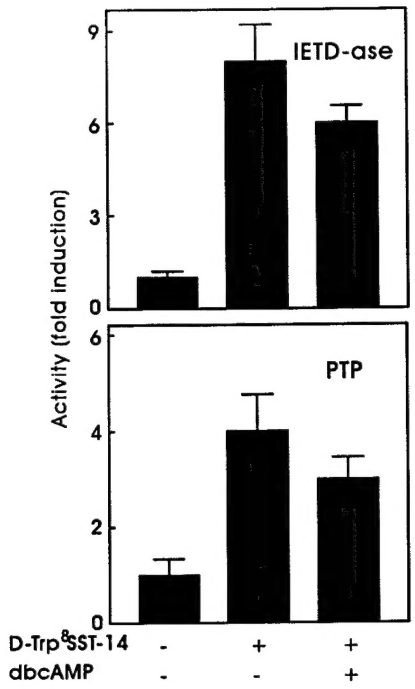

Figure 6

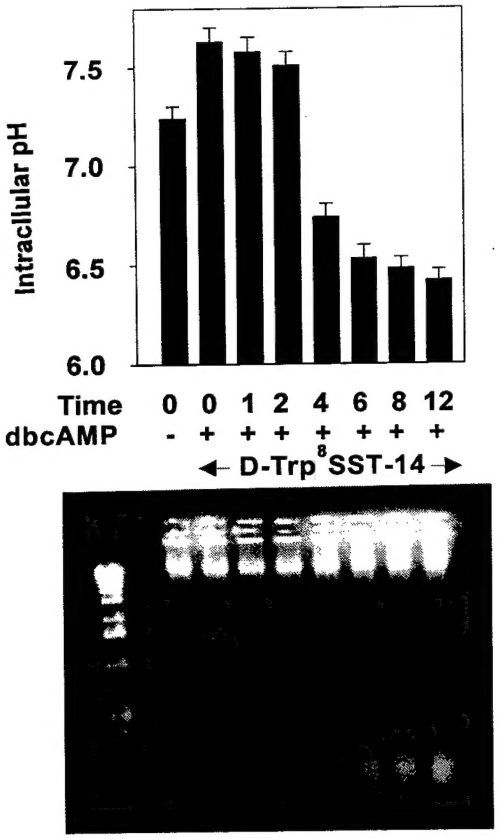

Figure 7

Figure 5: SST-induced apoptosis in MCF-7 cells is prevented by both VIP receptor-mediated and forskolin-induced increase in intracellular cAMP. Cells were treated with the reagents for 24 h, DNA extracted and analysed for the presence of oligonucleosomal degradation.

Figure 6: SST-induced activation of caspase-8 and protein tyrosine phosphatase (PTP) is not inhibited by dbcAMP. Cells were incubated for 4 h with 100 nM D-Trp8 SST-14 in the absence and presence of 50 μM dbcAMP. Caspase-8 activity was measured using IETD-AMC as the substrate while PTP activity was assayed using p-nitrophenyl phosphate as the substrate (mean ± SE, n=4).

Figure 7: Effect of timed addition of dbcAMP on SST-induced acidification and apoptosis in CHO-K1/hSSTR3 cells. Addition of dbcAMP inhibited SST-induced apoptosis by blocking SST-induced acidification (top panel). Addition of dbcAMP after 4 h during SST treatment failed to reverse SST-induced acidification and apoptosis (top and bottom panels).